Analysis of chromosomal radiosensitivity of healthy *BRCA2* mutation carriers and non-carriers in *BRCA* families with the G2 micronucleus assay

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Abstract. Breast cancer risk drastically increases in individuals with a heterozygous germline *BRCA1* or *BRCA2* mutation, while it is estimated to equal the population risk for relatives without the familial mutation (non-carriers). The aim of the present study was to use a G2 phase-specific micronucleus assay to investigate whether lymphocytes of healthy *BRCA2* mutation carriers are characterized by increased radiosensitivity compared to controls without a family history of breast/ovarian cancer and how this relates to healthy non-carrier relatives. *BRCA2* is active in homologous recombination, a DNA damage repair pathway, specifically active in the late S/G2 phase of the cell cycle. We found a significantly increased radiosensitivity in a cohort of healthy *BRCA2* mutation carriers compared to individuals without a familial history of breast cancer (P=0.046; Mann-Whitney U test). At the individual level, 50% of healthy *BRCA2* mutation carriers showed a radiosensitive phenotype (radiosensitivity score of 1 or 2), whereas 83% of the controls showed no radiosensitivity (P=0.038; one-tailed Fisher's exact test). An odds ratio of 5 (95% CI, 1.07-23.47) indicated an association between the *BRCA2* mutation and radiosensitivity in healthy mutation carriers. These results indicate the need for the gentle use of ionizing radiation for either diagnostic or therapeutic use in *BRCA2* mutation carriers. We detected no increased radiosensitivity in the non-carrier relatives.

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Introduction

BRCA1 and *BRCA2* heterozygous mutation carriers have a strongly increased risk to develop breast cancer (BC) and ovarian cancer (OC). The lifetime risk to develop BC is 70-80% for *BRCA1* mutation carriers and 50-60% for *BRCA2* mutation carriers (1). For relatives who did not inherit the germline *BRCA1/2* mutation segregating in the family (non-carrier relatives), the risk of BC occurrence is generally estimated to be as low as the risk assessed in the general population. This may imply that intensified BC detection screening, using, amongst others, mammography screening and MRI, as applied in individuals at high-risk is unnecessary in non-carriers (2-7). However, one study reported a 2-5-fold increase in BC occurrence in non-carriers of families with either *BRCA1* or *BRCA2* mutations (8). Another study reported a younger than expected age at diagnosis of BC for non-carriers, that was most evident in *BRCA1* families (9). Moreover, one study by Evans *et al* detected a possible higher relative risk for BC in non-carrier relatives of *BRCA2* families, compared to non-carriers in *BRCA1* families (10). In summary, these studies suggest that DNA alterations (for example SNPs) in other genes may modify the relative risk for the development of BC in non-carriers, compared to the general population. Moreover, the authors of these studies recommend targeted BC detection screening using for example mammography in non-carriers at a frequency comparable to the intensive BC screening performed in individuals at high-risk.

Both *BRCA1* and *BRCA2* are caretaker genes playing different roles in the repair of DNA double-strand breaks (DSB), induced by exposure to genotoxic agents such as ionizing radiation (IR). While *BRCA1* has a more general function in the detection and signaling of a DSB and in the activation of the G2/M cell cycle checkpoint, *BRCA2* exerts a specific function in the recruitment of RAD51 recombinase to the DSB site. This latter event is essential for the activation of the homologous recombination (HR) pathway, that relies on the undamaged sister chromatid as a template for resynthesis

of the damaged strand. This occurs in the late S and G2 phase of the cell cycle and leads to error-free repair of DSB (1).

Knowing that both *BRCA1* and *BRCA2* are important in the repair of DSB, exposure of mutation carriers to IR, a potent inducer of DSB, for either diagnostic or therapeutic purposes appears to be counterintuitive, as mutation carriers may be more prone to develop radiation-induced BC (11).

Radiosensitivity of *BRCA1* mutation carriers has previously been reported in the literature and was investigated and confirmed by our research group by means of the G2 micronucleus (MN) assay in combination with an evaluation of the G2/M checkpoint efficiency in peripheral blood lymphocytes of healthy *BRCA1* mutation carriers compared to healthy volunteers (12). However, the impact of IR on heterozygous cells of healthy *BRCA2* mutation carriers remains to be elucidated.

To date, several cohort studies were able to prove a positive correlation between exposure to diagnostic X-rays and BC risk in *BRCA2* mutation carriers (11,13,14). Others however, could not detect a similar correlation (15-18). Furthermore, Bernstein *et al* detected no increased induction of contralateral BC upon exposure to radiotherapy in *BRCA2* mutation carriers (19). Such discrepancies are likely due to differences in inclusion criteria, data acquisition and other issues of the studies. It is however difficult and unethical to design long-term unbiased studies to evaluate the relationship between *BRCA2* mutations, the exposure to diagnostic or therapeutic radiation and BC risk. *In vitro* chromosomal assays are effective tools to investigate radiosensitivity. Chromosomal radiosensitivity testing on lymphocytes from *BRCA2* mutation carriers has been performed with techniques such as the G0 MN and the G2 assays for chromatid breaks, occasionally enhanced with a whole-chromosome painting FISH (20-25). However, for several of these studies, it was unclear whether the *BRCA2* heterozygotes were healthy individuals or BC patients, which was previously broached by Baeyens *et al* (20). Furthermore, differences in the experimental setup make comparisons between studies difficult (26). Despite these differences, all but one study was able to detect an elevated chromosomal radiosensitivity in BC patients with a *BRCA2* mutation. However, no comparison was made with sporadic BC patients. The study of Baeyens *et al* previously demonstrated enhanced radiosensitivity in both BC patients with a *BRCA1/2* mutation and sporadic BC patients, suggesting that the enhanced sensitivity may not be the result of the mutation (20). No univocal results were achieved for healthy *BRCA2* mutation carriers. Radiosensitivity in non-carrier relatives has not been studied extensively, only one study reported no increased radiosensitivity measured with the G0 MN and G2 chromatid break assay in a small cohort (n=10) of relatives of both *BRCA1* and *BRCA2* families without the familial mutation when compared to a population cohort (20).

In the present study, we aimed to investigate chromosomal radiosensitivity in healthy *BRCA2* mutation carriers by means of the G2 MN assay. We previously used this assay and confirmed radiosensitivity in healthy *BRCA1* mutation carriers (n=18) compared to healthy controls without a family history of BC or OC (n=20) (12), and in an ataxia-telangiectasia patient and family members (27). In addition, we also included healthy relatives not carrying the familial germline *BRCA1* or *BRCA2* mutation in the present study. This cohort of non-carriers was included to evaluate radiosensitivity in individuals with a comparable genetic background, but without the familial *BRCA1* or *BRCA2* mutation.

Materials and methods

Sample collection. Blood samples were collected from individuals consulting the Centre for Medical Genetics of the Ghent University Hospital (CMG; Ghent, Belgium), in the context of predictive testing for hereditary BC. Heparin blood samples were collected for the G2 MN assay. In addition, EDTA samples were collected for mutation analysis. We collected blood samples from 18 *BRCA2* mutation carriers and 17 subjects from both *BRCA1* (n=9) and *BRCA2* (n=8) families not showing the familial mutation (non-carriers). None of the individuals selected for the present study had developed cancer at the time of the blood sample collection. We also selected 18 blood samples from a historical cohort of healthy volunteers without a personal or familial history of BC or OC for optimal age and gender match, to determine the normal distribution of MN yields in unaffected individuals from the general population (12).

The present study was approved by the Ethics Committee of Ghent University Hospital (B67020111641 d.d. 20/09/2011) and all participants signed an informed consent.

Molecular analysis. All healthy individuals selected for the present study had a family history of BC or OC and a mutation in either *BRCA1* or *BRCA2* was identified in each proband. All *BRCA2* mutation carriers are heterozygous for an unequivocal deleterious mutation. This was confirmed by Sanger sequencing of the relevant amplicon. Sanger sequencing was performed on the ABI3730XL instrument using the BigDye® Terminator Cycle Sequencing kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions; sequences were analyzed using the SeqPilot software (JSI Medical Systems GmbH, Ettenheim, Germany).

Molecular analyses were not performed in healthy volunteers due to the absence of a personal or familial anamnesis for BC or OC.

The G2 MN assay. The G2 MN assay was performed as previously described (12). In brief, heparinized blood was cultured in the presence of phytohaemagglutinin (PHA; 2% v/v; Gibco, Grand Island, NY, USA) to stimulate T-lymphocyte division. After 3 days, a population of cycling lymphocytes was obtained and the culture was irradiated with a 2 Gy dose of ^{60}Co γ-rays. We opted to use a dose of 2 Gy as this is a well-accepted dose for chromosomal radiosensitivity testing in lymphocytes (20-22,24). Immediately after irradiation, cytochalasin B (cyto B; 6 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to all cultures, including a non-irradiated culture. Cyto B blocks the cytokinesis and allows the identification of first-division cells as a binucleated (BN) cell. After an incubation period of 8 h, all cultures were fixed with the sequential addition of KCl (75 mM), a solution of methanol, acetic acid and Ringer (4:1:5), and a combination of methanol and acetic acid (4:1) to pelleted cells. Finally, the cell suspension was concentrated and spread on slides. Slides were stained with 4',6-diamidino-2-phenylindole (DAPI) and

scanned with a Metafer 4 platform and MN search software (MetaSystems GmbH, Altlussheim, Germany). The automated image analysis system selects BN cells and determines the number of MN/BN cells. BN cells and MN selection are manually checked for false positives or false negatives. For each condition, 2 cultures were prepared and 2 slides/culture were analyzed. A minimum of 600 BN cells were scored/coded slide. To assess individual radiosensitivity, a radiosensitivity score (RS score) was determined. The mean and SD of the MN yield of the group of healthy volunteers (HV) was set as the cut-off value to determine the RS score of HV, *BRCA2* mutation carriers and non-carrier relatives. An MN yield higher than the $mean_{HV}$ + $1SD_{HV}$ was scored as 1, indicating a milder radiosensitive phenotype, whereas a result higher than the $mean_{HV}$ + $2SD_{HV}$ was scored as 2, and indicated a more severe radiosensitive phenotype. When the individual value was lower than the $mean_{HV}$ + $1SD_{HV}$, a score of 0 was attributed to the tested subject.

Statistical analysis. Age and gender differences among the 3 groups were judged by means of a one-way ANOVA and Chi-square test, respectively. The median, interquartile range, average and standard deviation of micronuclei yields (number of MN/1,000 BN cells) were assessed in each group of subjects. Intergroup differences of MN yields between HV, *BRCA2* mutation carriers and non-carrier relatives of *BRCA1* and *BRCA2* pedigrees were analyzed by the Mann-Whitney-Wilcoxon test. A one-tailed Fisher's exact test was performed to compare the unpaired and independent proportion of patients showing a radiosensitive phenotype, evaluated by RS scoring. For both assays a 5% α error was set as the limit for statistical significance. The odds ratio (OR) was calculated, based on the RS scores in healthy individuals and *BRCA2* mutation carriers, to assess the association between the presence of a *BRCA2* mutation and radiosensitivity according to the following formula:

$$OR = \frac{\#\ BRCA2\ mutation\ carriers\ with\ an\ RS > 0\ x\ \#\ healthy\ volunteers\ with\ an\ RS = 0}{\#\ BRCA2\ mutation\ carriers\ with\ an\ RS = 0\ x\ \#\ healthy\ volunteers\ with\ an\ RS > 0}$$

The 95% confidence interval (CI) was used as a proxy for significance. The VassarStats platform and the SPSS software (IBM, version 23) were used to perform statistical analysis.

Results

The mean age did not significantly differ for the HV (35.3 years), the *BRCA2* mutation carriers (40.9 years) and the non-carrier relatives (40.0 years) (P=0.56; one-way ANOVA). In addition, no significant difference in gender distribution was observed for these 3 groups (68, 61 and 71% of the individuals were female, respectively) (P=0.84; Chi-square test). The number of spontaneously occurring micronuclei (MN yields in non-irradiated samples) was not significantly different among the 3 groups of enrolled subjects (Table I and Fig. 1).

Compared to HV without a family history of BC/OC, *BRCA2* mutation carriers showed a significant increase in mean MN yields after exposure to 2 Gy IR (P=0.046; Mann-Whitney).

Table I. Overview of the median, interquartile range, mean and SD of the micronucleus yield (#MN/1,000 BN).

Group data	0 Gy	2 Gy
Healthy volunteers (HV)		
Median	12	56
Interquartile range	9.75	27.5
Mean	14.33	61.22
SD	8.85	21.73
BRCA2 mutation carriers (MC)		
Median	14	74
Interquartile range	7.75	54.75
Mean	16.11	86.11
SD	6.91	41.87
P-value vs. healthy volunteers (Mann-Whitney)	0.177	0.046
Relatives who did not inherit the familial *BRCA1/2* mutation		
Median	16	69
Interquartile range	8	26
Mean	17.23	68.11
SD	7.74	22.30
P-value vs. healthy volunteers (Mann-Whitney)	0.116	0.400

SD standard deviation, MN micronucleus, BN binucleated cells.

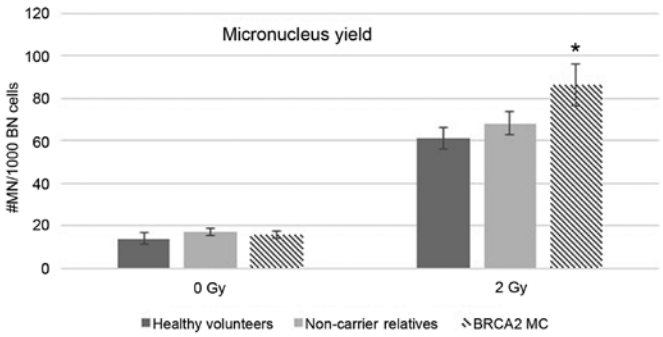

Figure 1. Mean G2 micronucleus (MN) yield. Mean MN yield for healthy volunteers, healthy relatives who did not inherit the familial *BRCA1/2* mutation and healthy *BRCA2* mutation carriers; *P<0.05 indicates a significant difference determined by Mann-Whitney U test. Error bars represent the standard error of the mean. BN, binucleated; MC, mutation carriers.

Conversely, the radiation-induced MN yields were similar in relatives who did not inherit the familial *BRCA1*/2 mutation and HV without a family history of BC/OC. The mean MN yield in *BRCA2* mutation carriers was higher compared to the mean yield in non-carriers (86.11 vs. 68.11 MN/1,000 BN cells, respectively). This difference, however, was not significant (P=0.298; Mann-Whitney), probably due to the small cohort and the high SD (Table I and Fig. 1). Furthermore, MN yields did not differ between non-carrier relatives from *BRCA1* or *BRCA2* pedigrees (Table II).

Table II. Overview of median, interquartile range, mean and SD of the micronucleus yield (#MN/1,000 BN) for healthy relatives who did not inherit the familial germline *BRCA1/2* mutation.

Goup data	0 Gy	2 Gy
Relatives who did not inherit the familial *BRCA1* mutation (n=9)		
Median	14	66
Interquartile range	12	57
Mean	16.44	69.04
SD	6.88	27.45
Relatives who did not inherit the familial *BRCA2* mutation (n=8)		
Median	16	70
Interquartile range	12.75	51.71
Mean	18.11	66.98
SD	9.18	16.59
P-value vs. *BRCA1* non-carriers (Mann-Whitney)	0.7339	0.9601

SD standard deviation, MN micronucleus, BN binucleated cells.

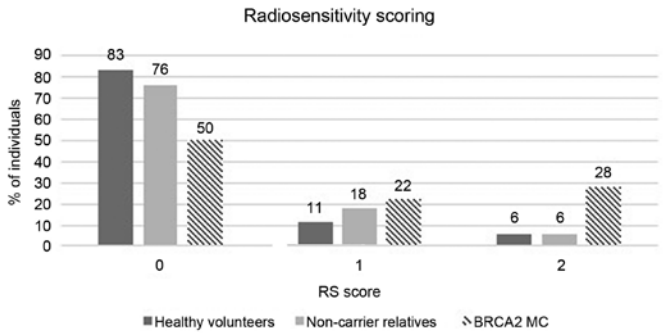

Figure 2. Radiosensitivity scoring. Distribution (%) of healthy volunteers, healthy relatives who did not inherit the familial *BRCA1/2* mutation and healthy *BRCA2* mutation carriers with different RS scores. RS score 0 indicates no increased radiosensitivity, RS score 1 indicates a milder radiosensitive phenotype and RS score 2 reflects a more severe radiosensitive phenotype. RS, radiosensitivity.

The individual MN yields after exposure to 2 Gy and the RS score for each *BRCA2* mutation carrier, non-carrier relative and healthy volunteer group are listed in Table III. Furthermore, Table III shows mutational data (both nucleotide and protein nomenclature) and individuals with the same family ID are related. Fig. 2 shows the distribution of the 3 groups for the different RS scores. A significantly higher number of *BRCA2* mutation carriers (n=9/18; 50%) showed increased RS scores (score 1 or 2) compared to HV (n=3/18; 17%) (P=0.038; one-tailed Fisher's exact test). For the relatives who did not inherit the familial germline mutation only 24% (n=4/17) showed an elevated radiosensitivity at the individual level. RS scoring in related individuals (see family ID in Table III) however shows some variation. An OR of 5 (95% CI, 1.07-23.46) for *BRCA2* mutation carriers vs. HV, indicates a significant association between the presence of a *BRCA2* mutation and radiosensitivity according to our criteria.

All but one of the 18 mutation carriers enrolled in the present study were heterozygous for a mutation predicted to result in a premature termination codon (PTC). The patient with the deleterious missense mutation [*BRCA2* c.8167G>C; p.(Asp2723His)] obtained an RS score of 2.

Discussion

Results of the G2 micronucleus (MN) assay performed after exposure to 2 Gy γ-rays showed a significantly increased radiosensitivity in healthy *BRCA2* mutation carriers compared to healthy controls. Previous studies with a large number of different techniques were able to demonstrate enhanced radiosensitivity in BC patients with a *BRCA2* mutation, however, no univocal results were achieved for healthy *BRCA2* mutation carriers (20-25). Non-carrier relatives of either *BRCA1* or *BRCA2* families did not show an increased radiosensitive phenotype compared to the cohort of healthy volunteers, which is in agreement with the study of Baeyens *et al* (20). We previously performed the G2 MN assay in a group of 18 healthy *BRCA1* mutation carriers, and found a significantly increased MN yield after exposure to 2 Gy γ-rays (12). These findings are analogous to the results of the present study, performed in healthy carriers of pathogenic *BRCA2* mutations. Fig. 3 shows the integration of the data from healthy *BRCA1* mutation carriers in the present study. The detection of an increased mean MN yield in both *BRCA1* and *BRCA2* mutation carriers after exposure to ionizing radiation can be explained by their mutual role in DNA double-strand break repair reviewed by Roy *et al* (1).

In our previous study we also analyzed the G2/M checkpoint activity by the addition of caffeine, an agent abrogating the G2/M checkpoint, to the irradiated cultures and demonstrated a significantly impaired checkpoint activation in *BRCA1* mutation carriers compared to healthy volunteers (12). Analysis of the G2/M checkpoint activation in the current *BRCA2* cohort did not reveal a significant difference (data not shown). This result is in agreement with the fact that BRCA2 is not activated in this particular checkpoint pathway as reviewed by Roy *et al* (1), but does not support the data obtained by Menzel *et al* (28), suggesting a role for BRCA2 as a regulator of G2 checkpoint maintenance following DNA damage introduced in a human osteosarcoma cell line (U2OS) expressing dominant-negative p53 by a high-dose of ionizing radiation (6 Gy).

The role of BRCA2 in the HR pathway, a DNA repair pathway active in the S and G2 phase of the cell cycle, is extensively reported in literature (1). The present study, focusing on radiosensitivity testing of lymphocytes in these phases of the cell cycle, showed an OR of 5 (95% CI, 1.07-23.47) for healthy individuals with a heterozygous *BRCA2* mutation compared to healthy controls. This indicates a positive association between the presence of a *BRCA2* mutation and radiosensitivity that could be attributed to deficient HR capacity in heterozygous cells.

Two independent research groups have reported a decreased DSB repair capacity in *BRCA2* heterozygous cells.

Table III. Germline mutation, family ID, micronucleus yields (#MN/1,000 BN) and RS score for *BRCA2* mutation carriers, relatives who did not inherit the familial mutation (non-carrier relatives) and healthy volunteers (numbering of the nucleotides according to RefSeq nr. NM_000059.3; A of ATG start codon=nucleotide +1).

	BRCA2 mutation carriers						Non-carrier relatives						Healthy volunteers			
ID	Family ID	Mutation: nucleotide	Mutation: protein	0 Gy	2 Gy	RS score	ID	Family ID	Family gene	0 Gy	2 Gy	RS score	ID	0 Gy	2 Gy	RS score
M2.01	BR-32-2170	c.658_659delGT	p.(Val220fs*4)	14	119	2	NM.06	BR-32-0156	BRCA2	17	51	0	D01	19	83	1
M2.02	BR-32-1748	c.1389_1390del	p.(Val464fs*3)	15	91	1	NM.17	BR-32-0342	BRCA1	8	29	0	D12	10	52	0
M2.03	BR-32-1748	c.1389_1390del	p.(Val464fs*3)	19	83	1	NM.01	BR-32-0645	BRCA1	20	63	0	D13	7	47	0
M2.04	BR-32-1748	c.1389_1390del	p.(Val464fs*3)	12	58	0	NM.10	BR-32-1134	BRCA1	14	74	0	D15	12	55	0
M2.05	BR-32-1748	c.1389_1390del	p.(Val464fs*3)	16	56	0	NM.13	BR-32-1225	BRCA1	18	43	0	D16	17	44	0
M2.06	BR-32-1758	c.1989del	p.(Phe663fs*5)	12	163	2	NM.12	BR-32-1225	BRCA1	11	57	0	D17	7	58	0
M2.07	BR-32-0884	c.4171del	p.(Glu1391fs*19)	37	65	0	NM.07	BR-32-1444	BRCA1	12	66	0	D21	13	48	0
M2.08	BR-32-0884	c.4171del	p.(Glu1391fs*19)	20	90	1	NM.08	BR-32-1444	BRCA1	12	78	0	D04	12	40	0
M2.09	BR-32-1759	c.4936_4939del	p.(Glu1646fs*23)	20	65	0	NM.02	BR-32-1494	BRCA1	24	87	1	D05	6	30	0
M2.10	BR-32-1759	c.4936_4939del	p.(Glu1646fs*23)	18	53	0	NM.16	BR-32-1967	BRCA1	29	125	2	D06	15	74	0
M2.11	BR-32-0156	c.6275_6276del	p.(Leu2092Profs*7)	12	63	0	NM.03	BR-32-0884	BRCA2	20	91	1	D29	9	29	0
M2.12	BR-32-1565	c.6275_6276del	p.(Leu2092Profs*7)	8	44	0	NM.04	BR-32-0884	BRCA2	16	73	0	D30	30	109	2
M2.13	BR-32-1930	c.6275_6276del	p.(Leu2092Profs*7)	23	183	2	NM.09	BR-32-1748	BRCA2	21	70	0	D32	7	96	1
M2.14	BR-32-1930	c.6275_6276del	p.(Leu2092Profs*7)	12	86	1	NM.11	BR-32-1758	BRCA2	8	45	0	D31	26	73	0
M2.15	BR-32-1920	c.8167G>C	p.(Asp2723His)	10	118	2	NM.05	BR-32-1759	BRCA2	38	85	1	D35	37	76	0
M2.16	BR-32-1628	c.8332-?_8487-?del	p.(Ile2778Lysfs*40)	22	29	0	NM.14	BR-32-1759	BRCA2	12	69	0	D37	6	75	0
M2.17	BR-32-0937	c.8904delC	p.(Val2969fs*7)	10	131	2	NM.15	BR-32-2170	BRCA2	13	52	0	D38	17	52	0
M2.18	BR-32-0082	c.9256+1G>C	r.9118_9256del; p.(Val3040Aspfs*18)	10	53	0							D39	8	61	0
Median				14.00	74.00					16.00	69.00			12.00	56.00	
Interquartile range				7.75	54.75					8.00	26.00			9.75	27.50	
Mean				16.11	86.11					17.23	68.11			14.33	61.22	
SD				6.91	41.87					7.74	22.30			8.85	21.73	

The splice site mutation present in M2.18 was previously described by Claes *et al* (39), where it was erroneously defined as IVS24G>A. SD standard deviation, MN micronucleus, BN binucleated cells, RS radiosensitivity.

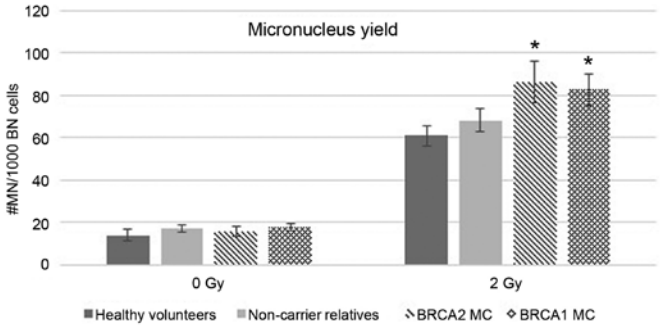

Figure 3. Mean G2 micronucleus yield. Mean MN yield for healthy volunteers, healthy relatives who did not inherit the familial *BRCA1/2* mutation, healthy *BRCA2* and healthy *BRCA1* mutation carriers; *P<0,05, indicates a significant difference determined by Mann-Whitney U test. Error bars represent the standard error of the mean. MN, micronucleus; BN, binucleated; MC, mutation carriers.

Keimling *et al* used an enhanced green fluorescent protein (EGFP)-based assay to report impaired HR capacity in lymphoblastoid cells with a *BRCA2* monoallelic truncating frameshift mutation. They confirmed this decrease in HR capacity in a BRCA2-knockdown HeLa cell line (29). Arnold *et al* demonstrated distinct defects in DNA DSB repair in lymphoblastoid cell lines (LCLs) from heterozygous *BRCA2* mutation carriers through analysis of γ-H2AX repair kinetics (30). Although, the latter study did not focus on DNA repair by HR, it indicates a malfunction of DSB repair in LCLs from *BRCA2* mutation carriers that could be attributed to diminished HR activity.

Most mutation carriers enrolled in the present study (n=17/18, 94%) had a mutation resulting in a premature termination codon (PTC). The presence of a PTC mutation is expected to activate nonsense-mediated decay of the gene transcript. Previous research from various groups including ours, demonstrated a reduction in mutant mRNA to approximately half of the WT mRNA levels in lymphocytes of individuals with a PTC mutation in *BRCA1* (12,31,32). Arnold *et al* (30) detected a similar mutant mRNA reduction for *BRCA2* mutations leading to a PTC. Furthermore, Arnold *et al* (30) and Keimling *et al* (29) report distinct reduced protein levels in LCLs from heterozygous *BRCA2* mutation carriers, although quantitative analysis of this variation was not performed. Previously, haploinsufficiency has been suggested as the mechanism for hereditary BC development in *BRCA1* and *BRCA2* mutation carriers (33). In the present study, a higher than expected number of radiosensitive individuals in the *BRCA2* mutation carriers indicates that haploinsufficiency may also be responsible for the radiosensitive phenotype in carriers of a mutation generating a PTC. In the present study, only one individual with a deleterious missense mutation was included. This substitution results in an amino acid change at position p.2723 and impairs protein functionality as shown by a homology-directed DNA break-repair functional assay (34). For this individual we obtained a high RS score of 2. Further research in larger patient cohorts with different types of mutations is needed to evaluate whether the type of mutation influences the radiosensitive phenotype or whether there are additional parameters determining this phenotype.

Results of the G2 MN assay showed no increased radiosensitivity in the group of non-carrier relatives of both *BRCA1* and *BRCA2* families compared to a group of healthy volunteers. Furthermore, only 24% of non-carriers showed an elevated radiosensitivity at the individual level (RS score 1 or 2). This was not significantly different from the fraction of healthy volunteers (17%) that was found to have an increased RS score. In addition, no difference was observed between non-carriers from *BRCA1* (RS score, 0 in 7/9 investigated relatives) or *BRCA2* families (RS score, 0 in 6/8 investigated relatives). However, we observed some variation within the different groups. We hypothesize that modifiers may play a role: indeed, selected SNPs in DNA-damage repair genes and other common variants have been associated with increased radiosensitivity (35-37) and increased BC risk (35,38). Further and larger studies are needed to evaluate the subtle influence of possible modifying factors on BC risk and radiosensitivity.

In conclusion, the present study demonstrated higher radiosensitivity in healthy *BRCA2* mutation carriers compared to healthy volunteers by means of the G2 MN assay after exposure of peripheral blood lymphocytes to a dose of 2 Gγ-rays. No increased radiosensitivity was observed in non-carrier relatives of *BRCA1* and *BRCA2* families. When evaluating radiosensitivity at the individual level, a significantly higher proportion of *BRCA2* mutation carriers (50%) showed a mild or more severe radiosensitivity compared to healthy volunteers (17%) and non-carriers (24%). Furthermore, an OR of 5 indicated a positive association between the *BRCA2* mutation and an increased radiosensitivity in healthy mutation carriers. These results indicate that care should be taken when applying ionizing radiation for either diagnostic or therapeutic purposes in *BRCA2* mutation carriers. However, a study including a larger population of subjects carrying different types of *BRCA2* mutations and non-carriers, must be performed to further elucidate the effect of each single mutation on the radiosensitive phenotype and the influence of possible underlying factors.

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